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⑤④ **CHEMICALLY MODIFIED GRANULOCYTE COLONY STIMULATING FACTOR.**

EP 0 401 384 A1 ⑤⑦ This invention provides a chemically modified protein prepared by combining polyethylene glycol with a polypeptide having substantially the following amino acid sequence and comprising the product of manifestation by a host cell of a foreign DNA sequence (1), wherein n is 0 or 1. This protein has a more lasting action of increasing neutrophils than that of the human granulocyte colony stimulating factor (G-CSF) known heretofore.

DESCRIPTION

CHEMICALLY-MODIFIED G-CSF

Technical Field

The present invention relates to a chemical modification of granulocyte colony-stimulating factor (G-CSF), by which chemical and/or physiological properties of G-CSF can be changed.

Background Art

Human G-CSF is one of haematopoietic growth factors. It has been shown to be present in the conditioned medium of a human bladder carcinoma cell line denominated 5637 (ATCC HT8-9) (Welte et al., Proc. Natl. Acad. Sci. (USA), 82, pp.1526-1530, (1985)). The determination of a DNA sequence encoding human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) has enabled the production of human G-CSF by means of recombinant genetic techniques.

Human G-CSF may be useful in the treatment of general haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in bone marrow transplantation. Wound healing burn treatment and the treatment of bacterial inflammation may also benefit from the application of human G-CSF (Welte et al., supra.).

It is generally observed that physiologically-active proteins administered into body can show their pharmacological activity only for a short period of time due to their high clearance rate in body. Furthermore, high

hydrophobicity of the proteins would reduce their stability.

For the purpose of decreasing the clearance rate, improving in stability or abolishing antigenicity of the proteins, some methods have been proposed wherein the proteins are chemically modified by using polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 289522/87, for EXAMPLE, discloses the reduction in immunogenicity of TNF which has been modified by polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 503171/87 discloses with respect to IL-2 and IFN- β the reduction in immunogenicity and aggregating property in an aqueous solution, and the prolongation of half-life in blood. In addition, there are disclosed the prolongation of half-life in blood and the disappearance of antigenicity or immunogenicity owing to the modification by polyethylene glycol with respect to a plasminogen activator (Japanese Patent Application Laying Open KOHYO No. 60938/88), IL-2, IFN- γ and SOD (Japanese Patent Application Laying Open KOHYO No. 10800/88), and IAP (Japanese Patent Application Laying Open KOHYO No. 126900/88).

However, these prior arts have not disclosed an improvement in biological activity and pharmacokinetics, which may be expected as a result of the modification of human G-CSF by polyethylene glycol.

Accordingly, it has been desired to prolong the half-life of human G-CSF in body so as to enhance its

lasting effect, as may be expected. Furthermore, G-CSF which may accelerate to recover from neutropenia sooner has been desired.

Disclosure of Invention

After vigorous investigations in order to solve the above problems, the present inventors have now found that the above desire can be realized by binding polyethylene glycol to human G-CSF, and have completed the present invention..

Any purified and isolated human G-CSF which is produced by host cells such as E. coli and animal cells transformed by using recombinant genetic techniques may be used in the present invention.

Among them, the human G-CSF which is produced by the transformed E. coli is particularly preferable. Such human G-CSF may be obtained in large quantities with high purity and homogeneity and substantially has the following amino acid sequence:

(Het)n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

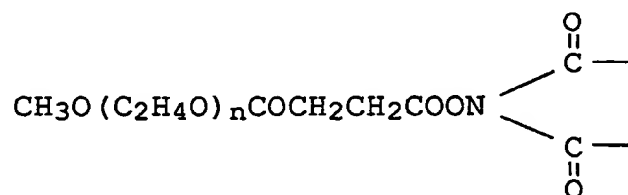
The above human G-CSF may, for example, be prepared according to a method disclosed in Japanese Patent Application Laying Open KOHYO No.500636/88. The wordings "substantially has the following amino acid sequence" mean that the above amino acid sequence may include one or more amino-acid changes (deletion, addition, insertion or replacement) as long as such changes will not cause any disadvantageous non-similarity in function to a naturally-occurring human G-CSF.

It is more preferable to use the human G-CSF substantially having the above amino acid sequence, in which at least one lysine, aspartic acid or glutamic acid residue is included.

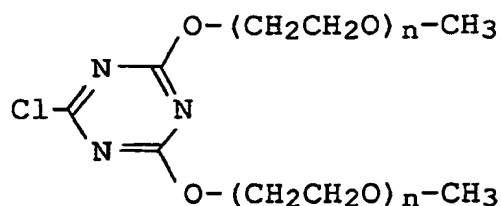
According to the present invention, polyethylene glycol is covalently bound through amino acid residues of the polypeptide of human G-CSF. The amino acid residue may be any reactive one having, for example, free amino or carboxyl groups, to which a terminal reactive group of an activated polyethylene glycol may be bound. The amino acid residues having the free amino groups may include lysine residues and N-terminal amino acid residue, and those having the free carboxyl group may include aspartic acid, glutamic acid residues and C-terminal amino acid residue.

A molecular weight of the polyethylene glycol used in the present invention is not restricted to any particular range, being, however, normally of from 500 - 20,000 and preferably of from 4,000 - 10,000.

Polyethylene glycol is bound onto human G-CSF via its terminal reactive group (or "a spacer"). Polyethylene glycol having the spacer is hereinafter referred to as "an activated polyethylene glycol". The spacer, for example, is that which mediates a bond between the free amino or carboxyl groups and polyethylene glycol. The activated polyethylene glycol which may be bound to the free amino group includes N-hydroxysuccinylimide polyethylene glycol having the following formula:

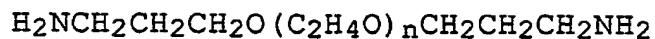


which may be prepared by activating succinic acid ester of polyethylene glycol with N-hydroxysuccinylimide. Another activated polyethylene glycol which may be bound to free amino group is 2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-s-triazine having the following formula:



which had been prepared by reacting polyethylene glycol monomethyl ether with cyanuric chloride. The activated polyethylene glycol which is bound to the free carboxyl

group includes polyoxyethylenediamine having the following formula:



The chemical modification through a covalent bond may be performed under any suitable condition generally adopted in a reaction of a biologically active substance with the activated polyethylene glycol. In case where the reactive amino acid residues in human G-CSF have the free amino groups, the above modification is preferably carried out in a buffer solution such as phosphate and borate (pH 7.5 - 10.0) for 1 - 5 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times, preferably 5 - 50 times the molar amount of the number of free amino groups of human G-CSF. On the other hand, in case where the reactive amino acid residues in human G-CSF have the free carboxyl groups, the above modification is preferably carried out in pH 3.5 - 5.5, for example, the modification with polyoxyethylenediamine is carried out in the presence of carbodiimide (pH 4.0 - 5.0) for 1 - 24 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times the molar amount of the number of free carboxyl groups of human G-CSF.

The extent of the modification of the amino acid residues may be optionally controlled depending on an amount of the activated polyethylene glycol used in the modification.

A polyethylene glycol-modified human G-CSF, namely chemically modified protein according to the present invention, may be purified from a reaction mixture by conventional methods which are used for purification of proteins, such as dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel chromatography and electrophoresis. Ion-exchange chromatography is particularly effective in removing unreacted polyethylene glycol and human G-CSF.

The present polyethylene glycol-modified human G-CSF has lasted its pharmacological effect, which may be possibly attributed to its prolonged half-life in body.

Furthermore, it is observed that the present polyethylene glycol-modified human G-CSF may accelerate the recovery from neutropenia.

The present polyethylene glycol-modified human G-CSF has essentially the same biological activity as an intact human G-CSF and may accordingly be used in the same application as that. The polyethylene glycol-modified human G-CSF has an activity for increasing the number of neutrophils, and it is therefore useful in the treatment of general haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in the treatment of infection and under receiving the therapy of bone marrow transplantation.

The present polyethylene glycol-modified human G-CSF may be formulated into pharmaceuticals containing also a pharmaceutically acceptable diluent, an agent for

preparing an isotonic solution, a pH-conditioner and the like in order to administer them into a patient.

The above pharmaceuticals may be administered subcutaneously, intramuscularly, intravenously or orally, depending on a purpose of treatment. A dose may be also changed on a kind and condition of the disorder of a patient to be treated, being normally between 0.1 μ g and 5 mg by injection and between 0.1 mg and 5 g in an oral administration for an adult .

Brief Description of Drawings

FIG.1 shows scanning patterns of PEG (4,500) G-CSF obtained by SDS-PAGE. The molar ratio of the activated PEG to the free amino groups of the human G-CSF is 0 for (a), 1 for (b), 5 for (c), 10 for (d) and 50 for (e), respectively. The peak of the intact human G-CSF is marked with *.

FIG.2 shows the time course of the change in number of neutrophils in mice after administration with human G-CSF or PEG-modified G-CSF. Each point represents an average value obtained from six mice with a standard deviation.

FIG.3 shows an accelerating effect of PEG-modified human G-CSF on the recovery from neutropenia induced by cyclophosphamide. Each point represents an average value obtained from six mice with a standard deviation.

FIG.4 shows an accelerating effect of PEG-modified G-CSF on the recovery from neutropenia induced by

5-FU. Each point represents an average value obtained from six mice with a standard deviation.

FIG.5 shows the results obtained in the study of half-life in serum of PEG (10,000) G-CSF (O) and human G-CSF (●). Each point represents an average value from three rats with a standard deviation.

Best Mode for Carrying Out the Invention

The present invention will be further illustrated by referring to the following EXAMPLEs which, however, are not be construed as limiting the scope of the present invention.

EXAMPLE 1Preparation of PEG (4,500) G-CSF

Recombinant human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) having the following amino acid sequence was used for the chemical modification according to the present invention:

Met

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met
Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
Arg Val Leu Arg His Leu Ala Gln Pro

As the activated polyethylene glycol (PEG) was used Methoxypolyethyleneglycol-Succinimydyl Succinate (Nippon Oil and Fats, Co., Ltd.) which had been prepared by activating a succinic acid ester of polyethylene glycol with an average molecular weight of about 4,500 with N-hydroxysuccinylimide.

The human G-CSF was incubated in 0.25 M sodium borate buffer (pH 8.0) for 1 hr at 4°C with the activated PEG in 1 - 50 times the molar amount of the number of the free amino groups in the human G-CSF. The resulting product was applied to Sephadex G25 which had been equilibrated with 10 mM NH_4HCO_3 for buffer-exchange, and then to DEAE ion-exchange chromatography so as to separate the PEG-modified human G-CSF from the agent and, if necessary, an unreacted human G-CSF. The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,500) G-CSF".

EXAMPLE 2

Characterization of PEG (4,500) G-CSF

PEG (4,500) G-CSF prepared in EXAMPLE 1 was characterized by the number of unmodified amino groups and a molecular weight estimated by SDS-PAGE.

The number of the unmodified amino groups was determined by reacting them with 0.1% TNBS in 4% NaHCO_3 followed by measurement of absorbance at 335 nm (Habeeb et al., Anal. Biochem., 14, pp.328-336, (1966)).

The molecular weight of PEG (4,500) G-CSF was determined by SDS-PAGE (16% gel, CBB staining) according to a method of Laemli, Nature, 227, p.680, 1970. Each lane on

the gel was scanned by using a chromato-scanner (SHIMADZU CORPORATION: CS-930) after staining.

When a molar ratio of the activated PEG to the number of free amino groups of human G-CSF increased, the extent of the modification also increased. The product prepared in said molar ratio of 1 has in addition to a band corresponding to an intact human G-CSF (19 K) another band with an apparent molecular weight of about 26 K (FIG. 1). With respect to the product prepared in the molar ratio of 5 or more, a band with a higher molecular weight was observed besides the above two bands. By scanning the resulting gel, a content of each band was determined. From the result in TABLE 1, it is estimated that the band of 26 K consists of human G-CSF wherein one human G-CSF molecule is bound with one activated PEG molecule and that a band of 34 K consists of human G-CSF wherein one human G-CSF molecule is bound with two activated PEG molecules.

TABLE 1 Characterization of PEG (4,500) G-CSF

PEG/NH ₂	Distribution			Modified NH ₂ (%)	Unmodified NH ₂ (an average number)
	19K	26K	34K		
1	86	12		5	4.8
2	68	31	1	15	4.3
3	56	42	2	15	4.3
4	36	48	16	20	4.0
5	31	49	20	27	3.7
6	25	50	25	27	3.7
7	20	50	28	27	3.7

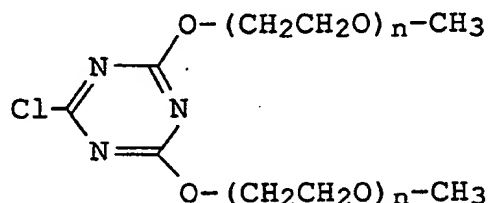
It was found that based on patterns obtained by SDS-PAGE of the fractions from the ion-exchange chromatography (shown in FIG.1) that the human G-CSF with a higher modification extent was eluted faster from a column and that the fraction finally eluted therefrom contained the intact human G-CSF.

The scanning patterns by SDS-PAGE of PEG (4,500) G-CSFs including those obtained with a higher molar ratio of PEG/NH₂ are shown in FIG.1.

EXAMPLE 3

Preparation of PEG (10,000) G-CSF

The same human G-CSF as used in EXAMPLE 1 was modified by an activated polyethylene glycol (an activated PEG 2; Seikagaku Kogyo K.K.) with a molecular weight of about 10,000 having the following formula:



which had been prepared by reacting polyethylene glycol monomethyl ether with cyanuric chloride.

The human G-CSF was incubated with the activated PEG 2 of 5 times of the molar of the number of the free amino groups of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 1 hr at room temperature. The resulting product was applied to Sephadex G25 which had been equilibrated with 10 mM NH₄HCO₃ for buffer-exchange,

and then to DEAE ion-exchange chromatography to separate the PEG-modified human G-CSF from an unreacted human G-CSF and reagent. The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 45 K with distributed among 30 K (10%), 40 K (70%) and 66 K (20%). The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (10,000) G-CSF".

Moreover, the human G-CSF was incubated with the activated PEG 2 of 10 times of the molar of the number of free amino groups of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 2 hrs at room temperature. The resulting product was subjected to separation in the same manner as stated above.

It is estimated in the same manner as in EXAMPLE 2 that the product of 30 K consists of human G-CSF wherein one human G-CSF molecule is coupled with one activated PEG molecule.

Furthermore, the human G-CSF was incubated with the activated PEG 2 of 50 times of the molar amount of the number of free amino groups of the human G-CSF.

The estimation of a molecular weight of the resulting products by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 51 K with distributed among 40 K (58 %) and 66 K (42 %).

EXAMPLE 4

Preparation of PEG (4,000) G-CSF

PEG-modified human G-CSF was prepared by covalently binding an activated polyethylene glycol, or polyoxyethylenediamine with an average molecular weight of 4,000 (Nippon Oil and Fats Co., Ltd.) to the above human G-CSF through the free carboxyl group thereof.

The human G-CSF and the activated polyethylene glycol of 60 times of the molar of the number of the free carboxyl groups of the human G-CSF were incubated in the presence of 0.05 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at a room temperature for overnight. The reaction was terminated by adding 1 M sodium acetate (pH 4.75) and further incubated at 25°C in the presence of 0.5 M hydroxyamine for 5 hrs in order to regenerate tyrosine residues. The resulting product was subjected to gel chromatography on TSK G3000SW which had been equilibrated with 10 mM sodium acetate (pH 5.5) to separate the PEG-modified human G-CSF from an unreacted human G-CSF and reagent. The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its molecular weight is distributed among 27 K (70%), 35 K (20%) and 42 K (10%). The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,000) G-CSF".

EXAMPLE 5

In vivo biological assay of PEG (4,500) G-CSF

Male ICR mice (Experiment I: 4 weeks old, Experiment II: 8 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF obtained

in EXAMPLE 1. Samples of the intact human G-CSF and PEG (4,500) G-CSF were intravenously injected into mice at a dose of 10 µg or 100 µg protein/kg. At 24 hrs (10 µg protein/kg) or 32 hrs (100 µg protein/kg) after the injection, blood was collected from orbital vein and leukocytes were counted by an auto blood cell counter E-2000 (Toa Medical Electronics, Japan). At the same time, blood smear was subjected to Wright-Giemsa stain and leukocytes fraction was determined by an auto blood cell analyzer MICROX (OMRON TATEISI ELECTRONICS CO.) to count the number of neutrophils. The results are summarized in TABLE 2 below.

In TABLE 2, PEG (4,500) G-CSF (1) is a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was five (FIG.1, C), PEG (4,500) G-CSF (2) is a 26 K fraction obtained from DEAE ion-exchange chromatography, and PEG (4,500) G-CSF (3) is a high molecular fraction (26 K:14%, 34 K:55%, >34 K:28%) obtained from said DEAE ion-exchange chromatography.

From the above results, it is observed that the number of neutrophils in the mice injected with PEG (4,500) G-CSFs (1), (2) and (3) have been much more increased than those in the mice injected with the intact G-CSF. Especially, PEG (4,500) G-CSFs (1) and (3) with a higher extent of the modification showed a remarkable increase in the number of neutrophils.

When human G-CSF is injected into mice at a dose of 10 µg protein/kg, the number of neutrophils increases,

and generally at 6 - 12 hrs after the injection, it gets to the maximum. After that, the number of neutrophils decreases slowly to a basal level about 30 hrs after injection. When 10 µg protein/kg injection, 24 hrs corresponds to the time span as normally required for the number of neutrophils which has once increased to again decrease almost to a basal level. In the case of 100 µg protein/kg injection, based on the above, the time for collection of blood (32 hrs after the injection) was determined. Accordingly, the above result that the numbers of neutrophils in the mice injected with PEG (4,500) G-CSFs (1), (2) and (3) are higher than those in the mice injected with the intact hG-CSF may indicate that the activity of human G-CSF in mice has been lasted by the present modification.

A mixture of human G-CSF and PEG did only show the same result as the intact human G-CSF (Data are not shown).

TABLE 2 Pharmacological activity (in vivo) of PEG-modified human G-CSF

Group	N	neutrophils ($\times 10^2/\mu\text{l}$)	Ratio (to vehicle)
<u>a. 10 $\mu\text{g/kg}$</u>			
<Exp. I >			
vehicle	5	5.6 ± 1.0	1.0
control G-CSF	6	9.6 ± 1.4	1.7
PEG(4500) G-CSF(1)	6	20.8 ± 2.6	3.7
PEG(4500) G-CSF(2)	6	17.5 ± 3.0	3.1
<Exp. II >			
vehicle	6	12.3 ± 1.7	1.0
control G-CSF	6	27.1 ± 4.6	2.2
PEG(4500) G-CSF(3)	6	54.0 ± 7.2	4.4
<u>b. 100 $\mu\text{g/kg}$</u>			
<Exp. I >			
vehicle	6	6.6 ± 0.7	1.0
control G-CSF	6	18.5 ± 2.3	2.8
PEG(4500) G-CSF(1)	6	42.9 ± 4.3	6.5
PEG(4500) G-CSF(2)	6	22.6 ± 1.9	3.4

EXAMPLE 6In vivo biological assay of PEG (4,000) G-CSF

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,000) G-CSF obtained in EXAMPLE 4. Samples of the intact human G-CSF and PEG (4,000) G-CSF were intravenously injected into mice at a dose of 10 μ g protein/kg. At 24 hrs after the injection, blood was collected from orbital vein and the number of neutrophils was counted as in EXAMPLE 5. The results are shown in TABLE 3.

It has been revealed that PEG (4,000) G-CSF in which the activated PEG is bound through the free carboxyl group has also increased the number of neutrophils more than the intact human G-CSF has.

TABLE 3 Pharmacological activity (in vivo) of PEG (4,000) G-CSF

Group	Number of Animals	Number of Neutrophils ($\times 10^2$ / μ l)	Ratio (to vehicle)
Vehicle	6	10.9 + 1.0	1.0
G-CSF (control)	6	16.4 + 1.4	1.5
PEG (4,000) G-CSF	6	23.3 + 2.5	2.1

EXAMPLE 7Increasing Effects of PEG-modified human G-CSFs on mice neutrophils

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF and PEG (10,000) G-CSF obtained in EXAMPLES 1 and 3, respectively. PEG (4,500) G-CSF used here is a high molecular fraction from DEAE ion-exchange chromatography of a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was fifty (an average molecular weight of 60K; 38K:20%, 58K:54%, 80K:27%). Samples of the human G-CSF, PEG (4,500) G-CSF and PEG (10,000) G-CSF were intravenously injected into mice at a dose of 10 µg protein/kg. At 6, 24, 32, 48 and 72 hrs after the injection, blood was collected from orbital vein and the number of neutrophils was counted as in EXAMPLE 5, except for using an auto blood cell counter CC180-A (Toa Medical Electronics, Japan).

As shown in FIG.2, in the case of the intact human G-CSF, the number of neutrophils decreases to a basal level 24 hrs after the injection. On the other hand, a significant increase of neutrophils was observed over 32 hrs and 48 hrs after the injection for PEG (4,500) G-CSF and PEG (10,000) G-CSF, respectively.

Moreover, male ICR mice (8 weeks old) were intravenously administered with the PEG (10,000) G-CSFs obtained in EXAMPLE 3; (a) an average molecular weight of 30 K, (b) an average molecular weight of 51 K; 40K:58%, 66K:42% at a dose of 10 µg protein/kg. At 24 hours after the injection the number of neutrophils was counted as in EXAMPLE 5. The results are shown in TABLE 4.

TABLE 4 Pharmacological activity (in vivo) of PEG (10,000) G-CSF

Group	Number of Animals	Number of Neutrophils ($\times 10^2$ / μ l)	Ratio (to vehicle)
Vehicle	5	7.4 + 0.6	1.0
G-CSF	5	16.4 + 3.1	2.2
PEG (10,000) G-CSF (a)	5	68.9 + 10.5	9.3
PEG (10,000) G-CSF (b)	5	95.8 + 6.4	12.9

Both PEG (10,000) G-CSF (a) and (b) have increased the number of neutrophils more than the intact human G-CSF has. Especially, PEG (10,000) G-CSF with a higher extent of the modification showed a more remarkable increase in the number of neutrophils, just like PEG (4,500) G-CSF did.

EXAMPLE 8

Effects of PEG-modified human G-CSF on cyclophosphamide-induced neutropenic mice

Male ICR mice (7 weeks old) were intraperitoneally injected with 200 mg/kg cyclophosphamide (CY) to induce neutropenia. Once a day for successive 4 days starting from one day after the CY injection, PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 were intravenously injected into the neutropenic mice at a dose of 10 μ g protein/kg. At 6, 24 and 48 hrs after the

last injection, blood was collected from orbital vein and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.3, PEG-modified G-CSFs have accelerate the recovery from neutropenia induced by the injection of cyclophosphamide similar or earlier than the intact G-CSF. Especially, PEG (10,000) G-CSF has effected a significant increase in the number of neutrophils.

EXAMPLE 9

Effects of PEG-modified human G-CSF on 5-FU-induced neutropenic mice

Female BDF₁ mice (7 weeks old, JAPAN SLC Co.,) were intravenously injected with 200 mg/kg 5-FU to induce neutropenia. At a dose of 10 µg protein/kg once a day for successive 11 days (PEG-1), for every other day (at day 1, 3, 5, 7, 9 and 11; PEG-2) and every third day (at day 1, 4, 7 and 10; PEG-3) starting from one day after the 5-FU injection, the same PEG (10,000) G-CSF as used in EXAMPLE 7 and the intact human G-CSF were subcutaneously injected into the neutropenic mice. At day 7, 8, 9, 10, 11, 12, 14 and 17, blood was collected from orbital vein and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.4, it took about 14 days to recover neutrophil counts of mice injected with only 5-FU to a basal level. On the other hand, it took about 11 days and 9 days to recover neutrophil counts of mice injected also with the intact human G-CSF, and PEG-1, 2 and 3, respectively. Thus, PEG-modified G-CSFs have accelerated the recovery from neutropenia induced by the injection of

5-FU earlier than the intact G-CSF. Moreover, even with fewer times of injection of the PEG-modified G-CSFs than the intact human G-CSF, the same phenomena as the above could be observed.

EXAMPLE 10

Acute toxicity of PEG-modified human G-CSF

Male and female Slc:IR mice (5 weeks old) groups consisting 6 mice each were intravenously administered with the same PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 as well as vehicles at a dose of 12 ml/kg. General conditions and survival of the treated mice were observed as often as possible for 6 hrs immediately after administration and once a day for the following 14 days. The body weight was checked at the day of injection, 5, 8, 12 and 15th days. Surviving mice were bled to death under ether anesthesia and subjected to pathologic autopsy.

As shown in TABLE 5, no mouse died for the observed period. LD 50 for both PEG (4,500) G-CSF and PEG (10,000) G-CSF was estimated over 3,000 µg protein/kg in both male and female mice. No remarkable change in general condition, body weight or opinion of the autopsy was observed for PEG (4,500) G-CSF or PEG (10,000) G-CSF. These results may suggest that the acute toxicity of PEG-modified human G-CSF is very weak, as the intact human G-CSF is .

TABLE. 5 Mortality of male and female mice

Sex	Compound	Dose ($\mu\text{g/kg}$)	Number of deaths on day															Mortality*	LD ₅₀ ($\mu\text{g/kg}$)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15 (Day)		
Male	Vehicle	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	—
	PEG4500-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
	PEG10000-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
Female	Vehicle	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	—
	PEG4500-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
	PEG10000-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000

* : No. of dead animals / No. of treated animals

EXAMPLE 11Determination of half-life of PEG-modified hG-CSF

Male Sprague-Dawley rats (7 weeks old) were used for study of pharmacokinetics of the intact human G-CSF and PEG (10,000) G-CSF prepared in EXAMPLE 3. Samples were intravenously injected into rats at a dose of 100 µg protein/kg. At 10 min, 2, 4, 8, 24 and 48 hrs after the injection, about 6 -7 ml of blood of each of three rats was collected from abdominal aorta into a polypropylene tube of about 15 ml volume and centrifuged (18,000 x g) at 4°C for 5 min to prepare a serum fraction. An amount of the active human G-CSFs contained in the serum fraction was determined by a bioassay for proliferation induction of mouse bone marrow cells on the basis of incorporation of ³H-thymidine (Ralph et al., Blood 66, pp.633-639, (1988)). The time course of serum concentration is shown in FIG. 5. The results indicate that the half lives of the intact human G-CSF and PEG (10,000) G-CSF are 1.79 hrs and 7.05 hrs, respectively, and AUCs are also 2,000 ng protein hrs/ml and 16,195 ng protein hrs/ ml, respectively. Accordingly, it is demonstrated that the clearance rate of PEG (10,000) G-CSF in the body has been decreased more than that of the intact human G-CSF has.

Industrial Applicability

It is expected that the present PEG-modified human G-CSF may make a great contribution to the treatment with human G-CSF because it has a neutrophils-increasing activity much more lasted than that of the intact human G-

CSF, enabling fewer numbers of administration with a lower dose.

CLAIMS

1. A chemically-modified protein prepared by binding polyethylene glycol to a polypeptide characterized by being the product of expression by a host cell of an exogenous DNA sequence and substantially having the following amino acid sequence:

(Met)_n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met
 Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro
 Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

2. The chemically-modified protein according to Claim 1 wherein polyethylene glycol is bound through an amino group of the amino acid(s) of the polypeptide.

3. The chemically-modified protein according to Claim 1 wherein polyethylene glycol is bound through a carboxyl group of the amino acid(s) of the polypeptide.

1/5

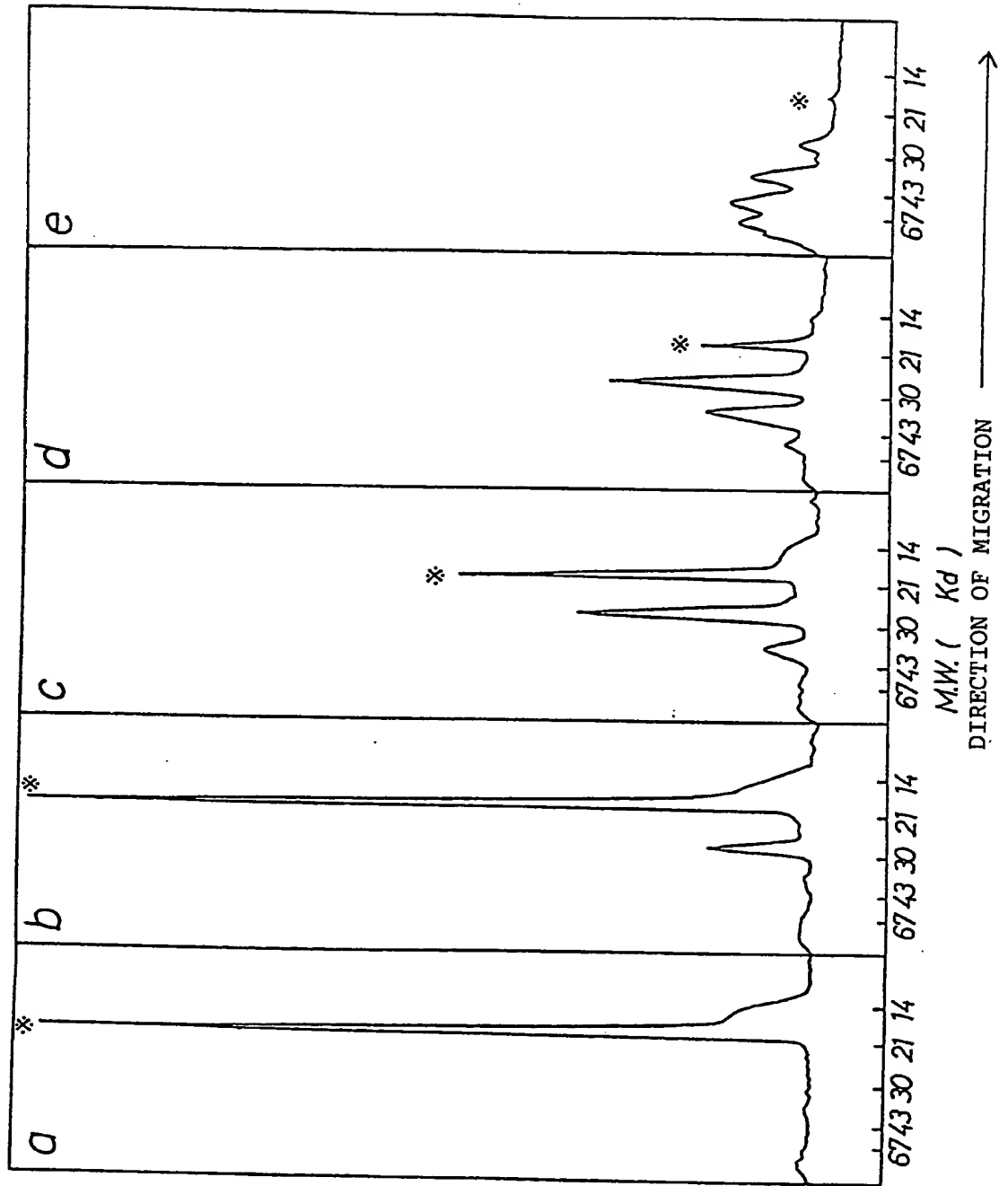


FIG. 1

O.D. (530 nm)

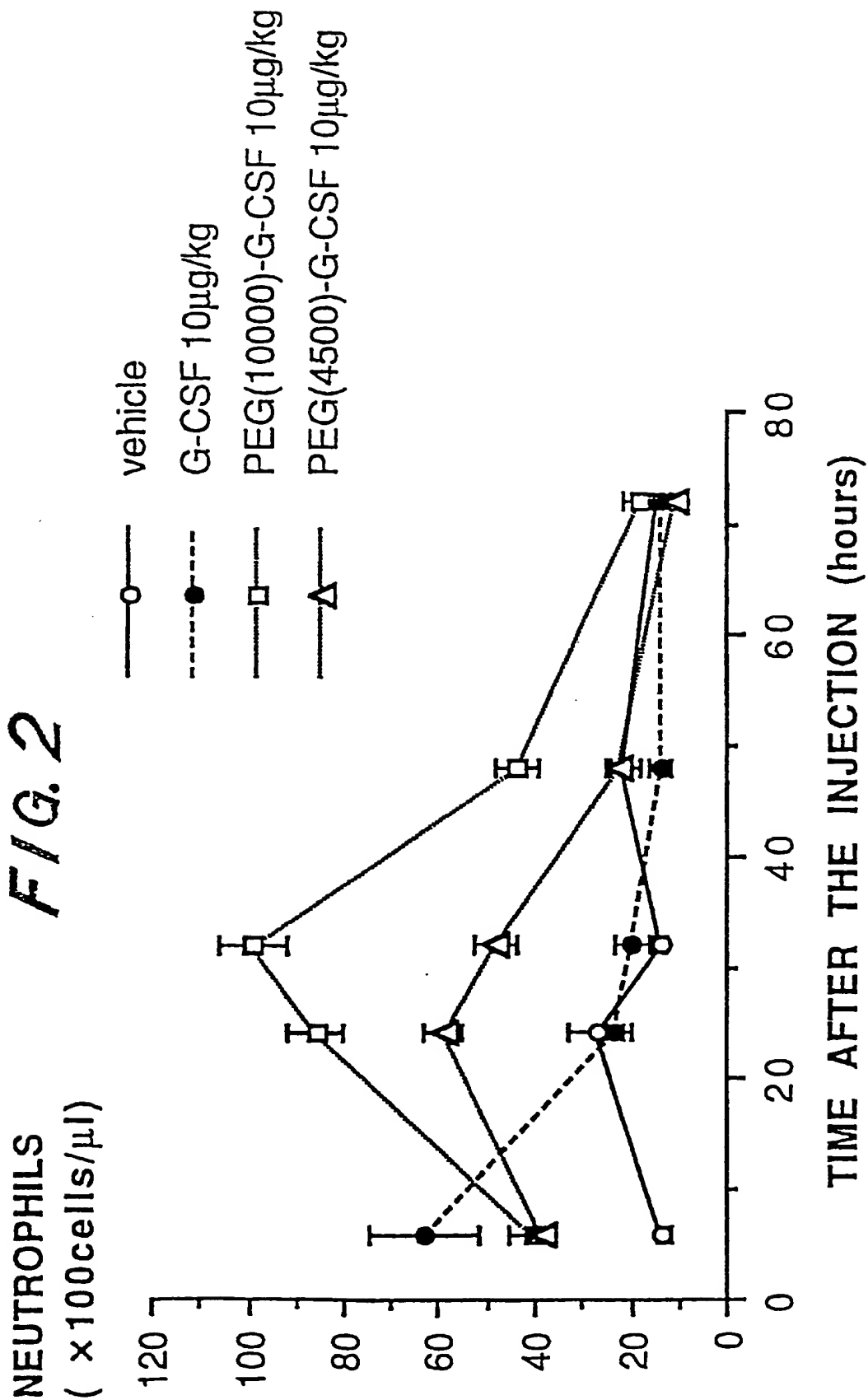
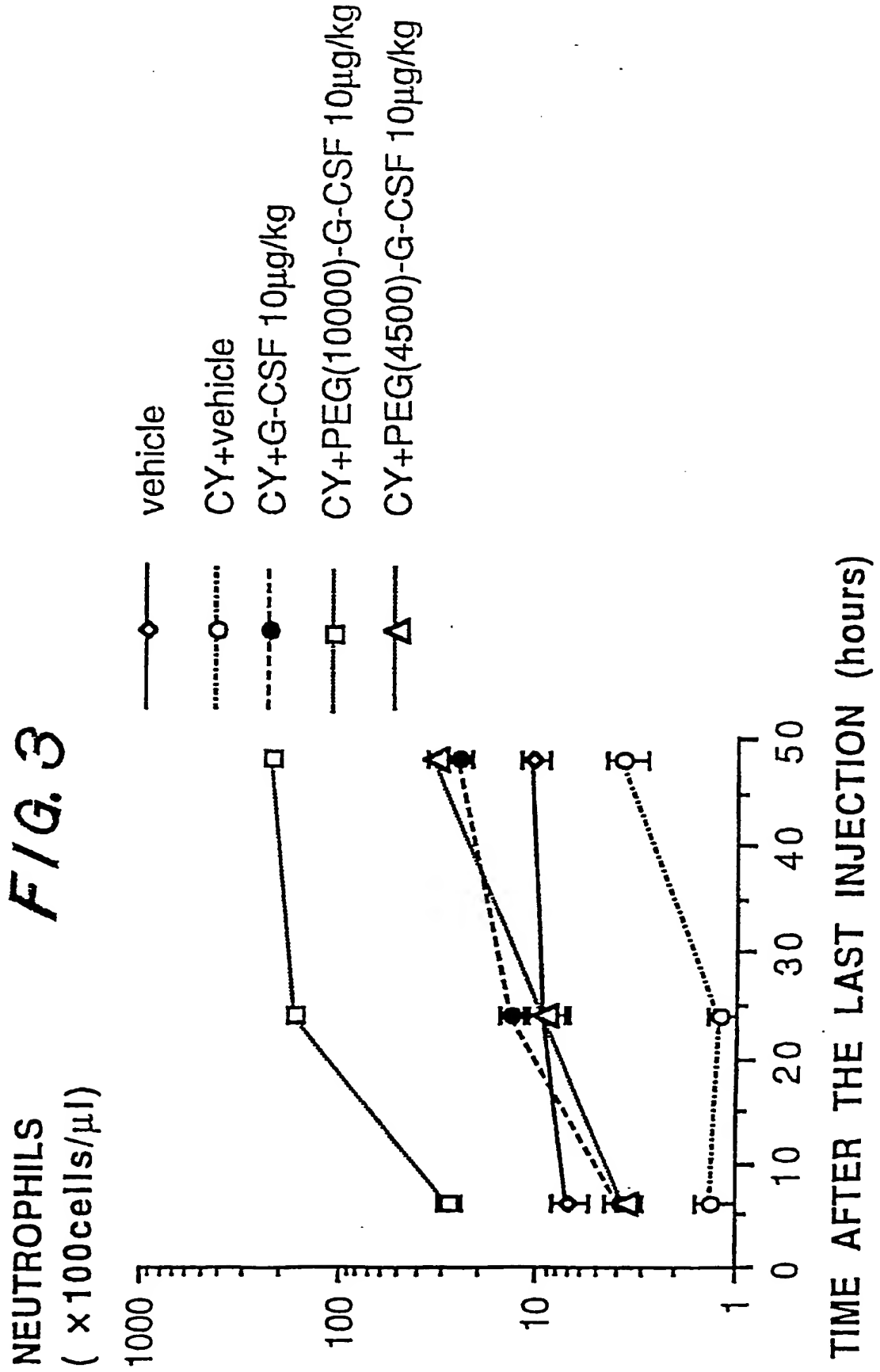
FIG. 2

FIG. 3



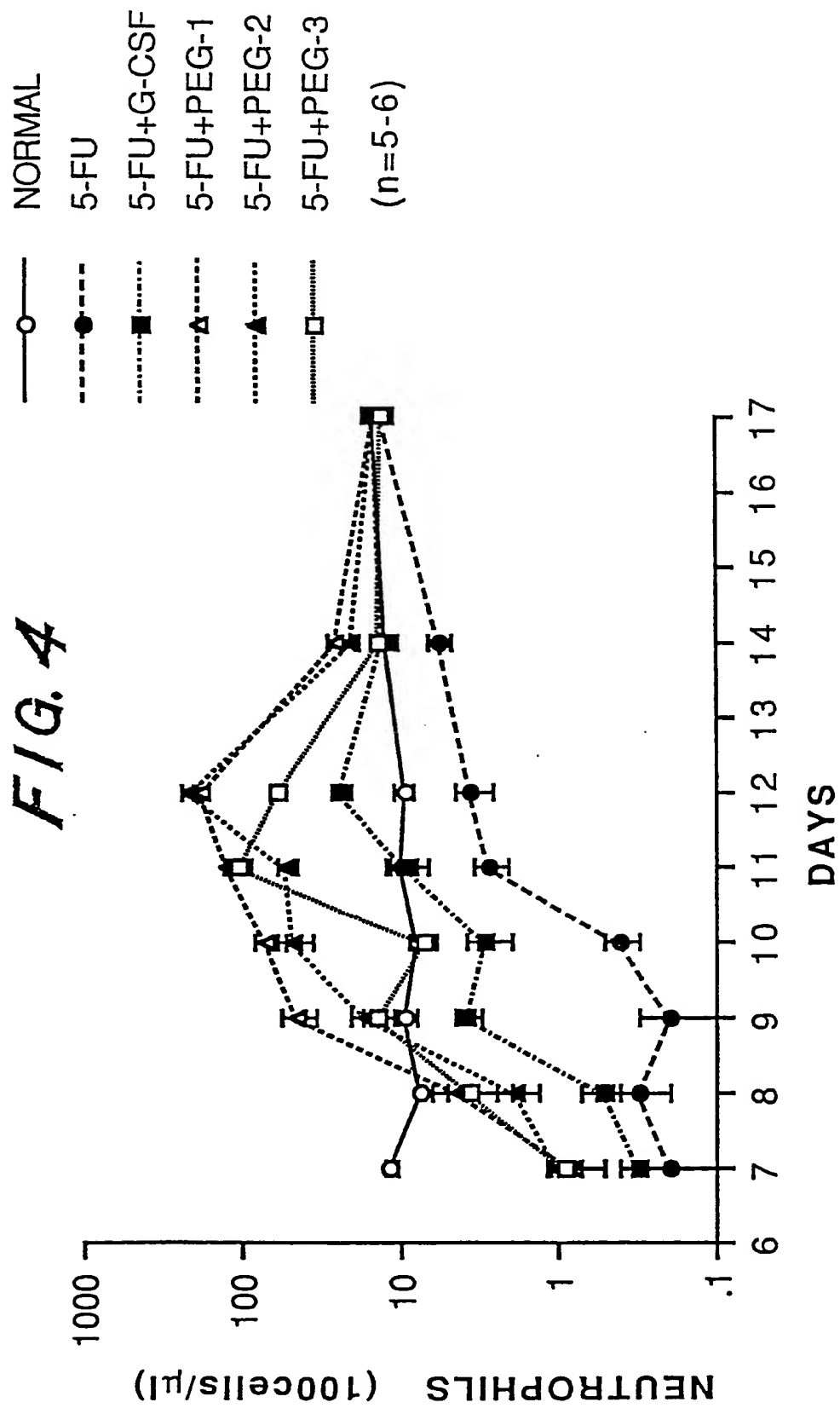
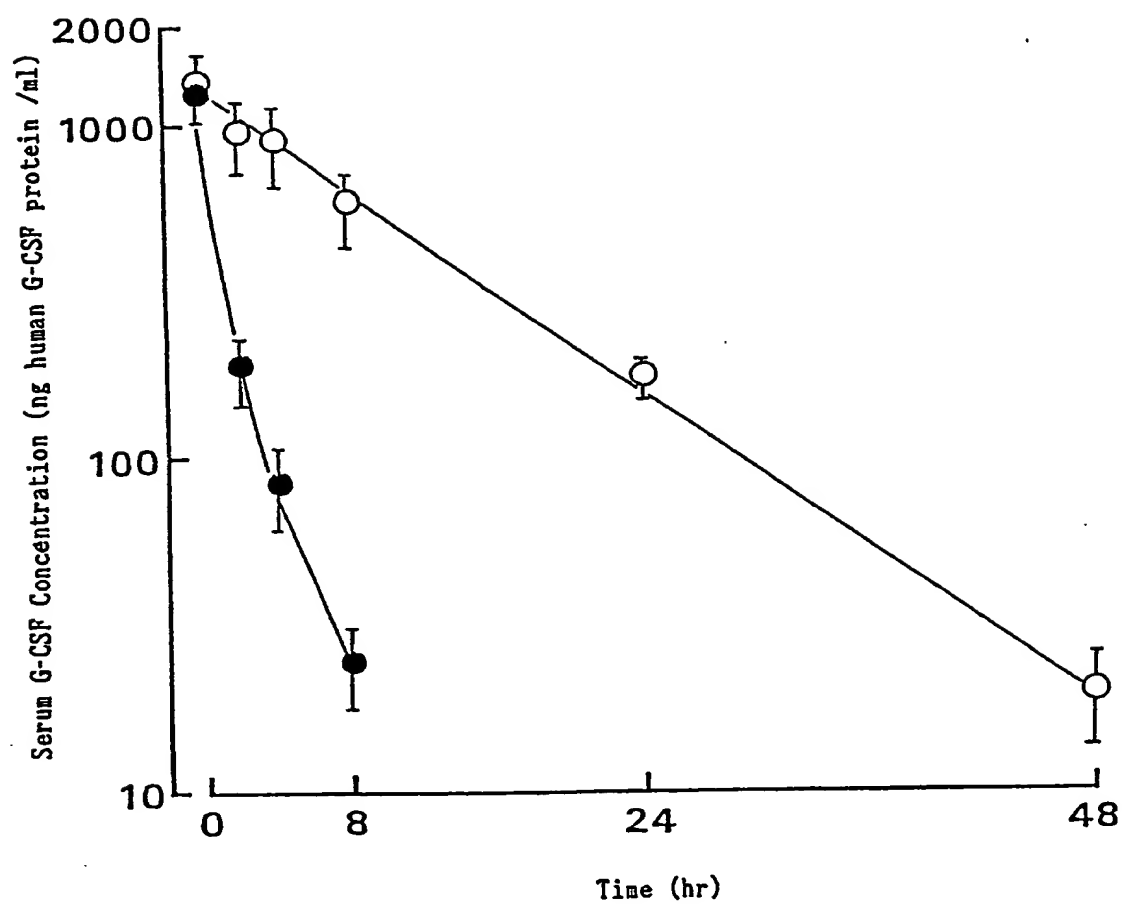


FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP89/01292

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl ⁵	C07K13/00, 3/08, A61K37/02	
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC	C07K13/00, 15/06, 3/08, 15/12, A61K37/02	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P	JP, A, 1-316400 (Kyowa Hakko Kogyo Co., Ltd.), 21 December 1989 (21. 12. 89), Pages 2 to 4 & EP, A, 335423 & AU, A, 8932341	1 - 2
X	JP, A, 63-10800 (Takeda Chemical Industries, Ltd.), 18 January 1988 (18. 01. 88), Pages 2 to 5 & EP, A, 236987	1 - 3
X	JP, A, 57-192435 (Toyobo Co., Ltd.), 26 November 1982 (26. 11. 82), Pages 2 to 3 (Family: none)	1 - 3
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
March 6, 1990 (06. 03. 90)		March 19, 1990 (19. 03. 90)
International Searching Authority		Signature of Authorized Officer
Japanese Patent Office		

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(87) International publication number:
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(54) **CHEMICALLY MODIFIED GRANULOCYTE COLONY STIMULATING FACTOR**
CHEMISCH MODIFIZIERTE GRANULOCYTENKOLONIE ERREGENDER FAKTOR
FACTEUR DE STIMULATION DE COLONIES DE GRANULOCYTES MODIFIES CHIMIQUEMENT

(84) Designated Contracting States:
AT BE CH DE ES FR GB IT LI LU NL SE

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12.12.1990 Bulletin 1990/50

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(56) References cited:
JP-A- 1 316 400 **JP-A- 6 310 800**
JP-A-57 192 435

• **No further relevant documents have been disclosed.**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description**CHEMICALLY-MODIFIED G-CSF**5 **Technical Field**

The present invention relates to a chemical modification of granulocyte colony-stimulating factor (G-CSF), by which chemical and/or physiological properties of G-CSF can be changed.

10 **Background Art**

Human G-CSF is one of haematopoietic growth factors. It has been shown to be present in the conditioned medium of a human bladder carcinoma cell line denominated 5637 (ATCC HT8-9) (Welte et al., Proc. Natl. Acad. Sci. (USA), 82, pp.1526-1530, (1985)). The determination of a DNA sequence encoding human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) has enabled the production of human G-CSF by means of recombinant genetic techniques.

Human G-CSF may be useful in the treatment of general haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in bone marrow transplantation. Wound healing burn treatment and the treatment of bacterial inflammation may also benefit from the application of human G-CSF (Welte et al., supra.).

It is generally observed that physiologically-active proteins administered into body can show their pharmacological activity only for a short period of time due to their high clearance rate in body. Furthermore, high hydrophobicity of the proteins would reduce their stability.

For the purpose of decreasing the clearance rate, improving in stability or abolishing antigenicity of the proteins, some methods have been proposed wherein the proteins are chemically modified by using polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 289522/87, for EXAMPLE, discloses the reduction in immunogenicity of TNF which has been modified by polyethylene glycol. Japanese Patent Application Laying Open KOHYO No. 503171/87 discloses with respect to IL-2 and IFN- β the reduction in immunogenicity and aggregating property in an aqueous solution, and the prolongation of half-life in blood. In addition, there are disclosed the prolongation of half-life in blood and the disappearance of antigenicity or immunogenicity owing to the modification by polyethylene glycol with respect to a plasminogen activator (Japanese Patent Application Laying Open KOHYO No.60938/88), IL-2, IFN- γ and SOD (Japanese Patent Application Laying Open KOHYO No.10800/88), and IAP (Japanese Patent Application Laying Open KOHYO No.126900/88).

However, these prior arts have not disclosed an improvement in biological activity and pharmacokinetics, which may be expected as a result of the modification of human G-CSF by polyethylene glycol.

Accordingly, it has been desired to prolong the half-life of human G-CSF in body so as to enhance its lasting effect, as may be expected. Furthermore, G-CSF which may accelerate to recover from neutropenia sooner has been desired.

Disclosure of Invention

After vigorous investigations in order to solve the above problems, the present inventors have now found that the above desire can be realized by binding polyethylene glycol to human G-CSF, and have completed the present invention..

Any purified and isolated human G-CSF which is produced by host cells such as E. coli and animal cells transformed by using recombinant genetic techniques may be used in the present invention.

Among them, the human G-CSF which is produced by the transformed E. coli is particularly preferable. Such human G-CSF may be obtained in large quantities with high purity and homogeneity and substantially has the following amino acid sequence:

(Het)n

5 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 10 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 15 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 20 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 25 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 30 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
 Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
 35 Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 40 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

45 The above human G-CSF may, for example, be prepared according to a method disclosed in Japanese Patent Application Laying Open KOHYO No.500636/88. The wordings "substantially has the following amino acid sequence" mean that the above amino acid sequence may include one or more amino-acid changes (deletion, addition, insertion or replacement) as long as such changes will not cause any disadvantageous non-similarity in function to a naturally-oc-
 50 ccurring human G-CSF.

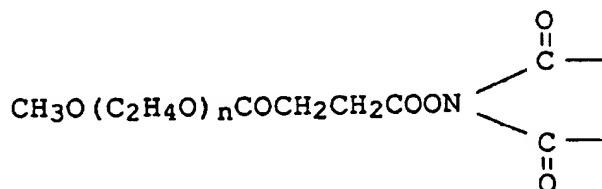
It is more preferable to use the human G-CSF substantially having the above amino acid sequence, in which at least one lysine, aspartic acid or glutamic acid residue is included.

According to the present invention, polyethylene glycol is covalently bound through amino acid residues of the polypeptide of human G-CSF. The amino acid residue may be any reactive one having, for example, free amino or
 55 carboxyl groups, to which a terminal reactive group of an activated polyethylene glycol may be bound. The amino acid residues having the free amino groups may include lysine residues and N-terminal amino acid residue, and those having the free carboxyl group may include aspartic acid, glutamic acid residues and C-terminal amino acid residue.

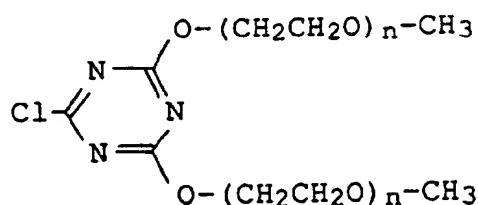
A molecular weight of the polyethylene glycol used in the present invention is not restricted to any particular range,

being, however, normally of from 500 - 20,000 and preferably of from 4,000 - 10,000.

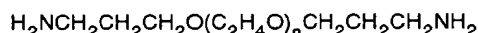
Polyethylene glycol is bound onto human G-CSF via its terminal reactive group (or "a spacer"). Polyethylene glycol having the spacer is hereinafter referred to as "an activated polyethylene glycol". The spacer, for example, is that which mediates a bond between the free amino or carboxyl groups and polyethylene glycol. The activated polyethylene glycol which may be bound to the free amino group includes N-hydroxysuccinylimide polyethylene glycol having the following formula:



which may be prepared by activating succinic acid ester of polyethylene glycol with N-hydroxysuccinylimide. Another activated polyethylene glycol which may be bound to free amino group is 2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-s-triazine having the following formula:



which had been prepared by reacting polyethylene glycol monomethyl ether with cyanuric chloride. The activated polyethylene glycol which is bound to the free carboxyl group includes polyoxyethylenediamine having the following formula:



The chemical modification through a covalent bond may be performed under any suitable condition generally adopted in a reaction of a biologically active substance with the activated polyethylene glycol. In case where the reactive amino acid residues in human G-CSF have the free amino groups, the above modification is preferably carried out in a buffer solution such as phosphate and borate (pH 7.5 - 10.0) for 1 - 5 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times, preferably 5 - 50 times the molar amount of the number of free amino groups of human G-CSF. On the other hand, in case where the reactive amino acid residues in human G-CSF have the free carboxyl groups, the above modification is preferably carried out in pH 3.5 - 5.5, for example, the modification with polyoxyethylenediamine is carried out in the presence of carbodiimide (pH 4.0 - 5.0) for 1 - 24 hrs at 4 - 37°C. The activated polyethylene glycol may be used in 1 - 200 times the molar amount of the number of free carboxyl groups of human G-CSF.

The extent of the modification of the amino acid residues may be optionally controlled depending on an amount of the activated polyethylene glycol used in the modification.

A polyethylene glycol-modified human G-CSF, namely chemically modified protein according to the present invention, may be purified from a reaction mixture by conventional methods which are used for purification of proteins, such as dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel chromatography and electrophoresis. Ion-exchange chromatography is particularly effective in removing unreacted polyethylene glycol and human G-CSF.

The present polyethylene glycol-modified human G-CSF has lasted its pharmacological effect, which may be possibly attributed to its prolonged half-life in body.

Furthermore, it is observed that the present polyethylene glycol-modified human G-CSF may accelerate the recovery from neutropenia.

The present polyethylene glycol-modified human G-CSF has essentially the same biological activity as an intact human G-CSF and may accordingly be used in the same application as that. The polyethylene glycol-modified human G-CSF has an activity for increasing the number of neutrophils, and it is therefore useful in the treatment of general haematopoietic disorders including those arising from chemotherapy or from radiation therapy. It may be also useful in the treatment of infection and under receiving the therapy of bone marrow transplantation.

The present polyethylene glycol-modified human G-CSF may be formulated into pharmaceuticals containing also

a pharmaceutically acceptable diluent, an agent for preparing an isotonic solution, a pH-conditioner and the like in order to administer them into a patient.

The above pharmaceuticals may be administered subcutaneously, intramuscularly, intravenously or orally, depending on a purpose of treatment. A dose may be also changed on a kind and condition of the disorder of a patient to be treated, being normally between 0.1 μ g and 5 mg by injection and between 0.1 mg and 5 g in an oral administration for an adult.

Brief Description of Drawings

FIG.1 shows scanning patterns of PEG (4,500) G-CSF obtained by SDS-PAGE. The molar ratio of the activated PEG to the free amino groups of the human G-CSF is 0 for (a), 1 for (b), 5 for (c), 10 for (d) and 50 for (e), respectively. The peak of the intact human G-CSF is marked with *.

FIG.2 shows the time course of the change in number of neutrophils in mice after administration with human G-CSF or PEG-modified G-CSF. Each point represents an average value obtained from six mice with a standard deviation.

FIG.3 shows an accelerating effect of PEG-modified human G-CSF on the recovery from neutropenia induced by cyclophosphamide. Each point represents an average value obtained from six mice with a standard deviation.

FIG.4 shows an accelerating effect of PEG-modified G-CSF on the recovery from neutropenia induced by 5-FU. Each point represents an average value obtained from six mice with a standard deviation.

FIG.5 shows the results obtained in the study of half-life in serum of PEG (10,000) G-CSF (O) and human G-CSF (●). Each point represents an average value from three rats with a standard deviation.

Best Mode for Carrying Out the Invention

The present invention will be further illustrated by referring to the following EXAMPLEs which, however, are not be construed as limiting the scope of the present invention.

EXAMPLE 1

Preparation of PEG (4,500) G-CSF

Recombinant human G-CSF (Japanese Patent Application Laying Open KOHYO No. 500636/88) having the following amino acid sequence was used for the chemical modification according to the present invention:

Het

5 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 10 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 15 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 20 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 25 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 30 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
 Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
 35 Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 40 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 Arg Val Leu Arg His Leu Ala Gln Pro

45 As the activated polyethylene glycol (PEG) was used Methoxypolyethyleneglycol-Succinimydyl Succinate (Nippon Oil and Fats, Co., Ltd.) which had been prepared by activating a succinic acid ester of polyethylene glycol with an average molecular weight of about 4,500 with N-hydroxysuccinylimide.

50 The human G-CSF was incubated in 0.25 M sodium borate buffer (pH 8.0) for 1 hr at 4°C with the activated PEG in 1 - 50 times the molar amount of the number of the free amino groups in the human G-CSF. The resulting product was applied to Sephadex G25 which had been equilibrated with 10 mM NH_4HCO_3 for buffer-exchange, and then to DEAE ion-exchange chromatography so as to separate the PEG-modified human G-CSF from the agent and, if necessary, an unreacted human G-CSF. The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,500) G-CSF".

EXAMPLE 2Characterization of PEG (4,500) G-CSF

PEG (4,500) G-CSF prepared in EXAMPLE 1 was characterized by the number of unmodified amino groups and a molecular weight estimated by SDS-PAGE.

The number of the unmodified amino groups was determined by reacting them with 0.1% TNBS in 4% NaHCO₃ followed by measurement of absorbance at 335 nm (Habeeb et al., Anal. Biochem., 14, pp.328-336, (1966)).

The molecular weight of PEG (4,500) G-CSF was determined by SDS-PAGE (16% gel, CBB staining) according to a method of Laemmli, Nature, 227, p.680, 1970. Each lane on the gel was scanned by using a chromat-scanner (SHIMADZU CORPORATION: CS-930) after staining.

When a molar ratio of the activated PEG to the number of free amino groups of human G-CSF increased, the extent of the modification also increased. The product prepared in said molar ratio of 1 has in addition to a band corresponding to an intact human G-CSF (19 K) another band with an apparent molecular weight of about 26 K (FIG. 1). With respect to the product prepared in the molar ratio of 5 or more, a band with a higher molecular weight was observed besides the above two bands. By scanning the resulting gel, a content of each band was determined. From the result in TABLE 1, it is estimated that the band of 26 K consists of human G-CSF wherein one human G-CSF molecule is bound with one activated PEG molecule and that a band of 34 K consists of human G-CSF wherein one human G-CSF molecule is bound with two activated PEG molecules.

TABLE 1

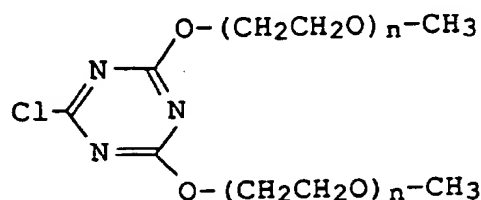
Characterization of PEG (4,500) G-CSF					
PEG/NH ₂	Distribution			Modified NH ₂ (%)	Unmodified NH ₂ (an average number)
	19K	26K	34K		
1	86	12		5	4.8
2	68	31	1	15	4.3
3	56	42	2	15	4.3
4	36	48	16	20	4.0
5	31	49	20	27	3.7
6	25	50	25	27	3.7
7	20	50	28	27	3.7

It was found that based on patterns obtained by SDS-PAGE of the fractions from the ion-exchange chromatography (shown in FIG. 1) that the human G-CSF with a higher modification extent was eluted faster from a column and that the fraction finally eluted therefrom contained the intact human G-CSF.

The scanning patterns by SDS-PAGE of PEG (4,500) G-CSFs including those obtained with a higher molar ratio of PEG/NH₂ are shown in FIG. 1.

EXAMPLE 3Preparation of PEG (10,000) G-CSF

The same human G-CSF as used in EXAMPLE 1 was modified by an activated polyethylene glycol (an activated PEG 2; Seikagaku Kogyo K.K.) with a molecular weight of about 10,000 having the following formula:



which had been prepared by reacting polyethylene glycol monomethyl ether with cyanuric chloride.

The human G-CSF was incubated with the activated PEG 2 of 5 times of the molar of the number of the free amino groups of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 1 hr at room temperature. The resulting product was applied to Sephadex G25 which had been equilibrated with 10 mM NH_4HCO_3 for buffer-exchange, and then to DEAE ion-exchange chromatography to separate the PEG-modified human G-CSF from an unreacted human G-CSF and reagent. The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 45 K with distributed among 30 K (10%), 40 K (70%) and 66 K (20%). The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (10,000) G-CSF".

Moreover, the human G-CSF was incubated with the activated PEG 2 of 10 times of the molar of the number of free amino groups of the human G-CSF in 0.25 M sodium borate buffer solution (pH 10.0) for 2 hrs at room temperature. The resulting product was subjected to separation in the same manner as stated above.

It is estimated in the same manner as in EXAMPLE 2 that the product of 30 K consists of human G-CSF wherein one human G-CSF molecule is coupled with one activated PEG molecule.

Furthermore, the human G-CSF was incubated with the activated PEG 2 of 50 times of the molar amount of the number of free amino groups of the human G-CSF.

The estimation of a molecular weight of the resulting products by SDS-PAGE as in EXAMPLE 2 has revealed that its average molecular weight is about 51 K with distributed among 40 K (58 %) and 66 K (42 %).

EXAMPLE 4

Preparation of PEG (4,000) G-CSF

PEG-modified human G-CSF was prepared by covalently binding an activated polyethylene glycol, or polyoxyethylenediamine with an average molecular weight of 4,000 (Nippon Oil and Fats Co., Ltd.) to the above human G-CSF through the free carboxyl group thereof.

The human G-CSF and the activated polyethylene glycol of 60 times of the molar of the number of the free carboxyl groups of the human G-CSF were incubated in the presence of 0.05 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at a room temperature for overnight. The reaction was terminated by adding 1 M sodium acetate (pH 4.75) and further incubated at 25°C in the presence of 0.5 M hydroxylamine for 5 hrs in order to regenerate tyrosine residues. The resulting product was subjected to gel chromatography on TSK G3000SW which had been equilibrated with 10 mM sodium acetate (pH 5.5) to separate the PEG-modified human G-CSF from an unreacted human G-CSF and reagent. The estimation of a molecular weight of the product by SDS-PAGE as in EXAMPLE 2 has revealed that its molecular weight is distributed among 27 K (70%), 35 K (20%) and 42 K (10%). The resultant PEG-modified human G-CSF is hereinafter referred to as "PEG (4,000) G-CSF".

EXAMPLE 5

In vivo biological assay of PEG (4,500) G-CSF

Male ICR mice (Experiment I: 4 weeks old, Experiment II: 8 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF obtained in EXAMPLE 1. Samples of the intact human G-CSF and PEG (4,500) G-CSF were intravenously injected into mice at a dose of 10 μg or 100 μg protein/kg. At 24 hrs (10 μg protein/kg) or 32 hrs (100 μg protein/kg) after the injection, blood was collected from orbital vein and leukocytes were counted by an auto blood cell counter E-2000 (Toa Medical Electronics, Japan). At the same time, blood smear was subjected to Wright-Giemsa stain and leukocytes fraction was determined by an auto blood cell analyzer MICROX (OMRON TATEISI ELECTRONICS CO.) to count the number of neutrophils. The results are summarized in TABLE 2 below.

In TABLE 2, PEG (4,500) G-CSF (1) is a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was five (FIG. 1, C), PEG (4,500) G-CSF (2) is a 26 K fraction obtained from DEAE ion-exchange chromatography, and PEG (4,500) G-CSF (3) is a high molecular fraction (26 K:14%, 34 K:55%, >34 K:28%) obtained from said DEAE ion-exchange chromatography.

From the above results, it is observed that the number of neutrophils in the mice injected with PEG (4,500) G-CSFs (1), (2) and (3) have been much more increased than those in the mice injected with the intact G-CSF. Especially, PEG (4,500) G-CSFs (1) and (3) with a higher extent of the modification showed a remarkable increase in the number of neutrophils.

When human G-CSF is injected into mice at a dose of 10 μg protein/kg, the number of neutrophils increases, and generally at 6 - 12 hrs after the injection, it gets to the maximum. After that, the number of neutrophils decreases slowly to a basal level about 30 hrs after injection. When 10 μg protein/kg injection, 24 hrs corresponds to the time span as normally required for the number of neutrophils which has once increased to again decrease almost to a basal level. In the case of 100 μg protein/kg injection, based on the above, the time for collection of blood (32 hrs after the injection)

was determined. Accordingly, the above result that the numbers of neutrophils in the mice injected with PEG (4,500) G-CSFs (1), (2) and (3) are higher than those in the mice injected with the intact hG-CSF may indicate that the activity of human G-CSF in mice has been lasted by the present modification.

A mixture of human G-CSF and PEG did only show the same result as the intact human G-CSF (Data are not shown).

TABLE 2

Pharmacological activity (in vivo) of PEG-modified human G-CSF			
Group	N	neutrophils ($\times 10^2/\mu\text{l}$)	Ratio (to vehicle)
a. 10 $\mu\text{g/kg}$			
<Exp. I>			
vehicle	5	5.6 \pm 1.0	1.0
control G-CSF	6	9.6 \pm 1.4	1.7
PEG(4500) G-CSF(1)	6	20.8 \pm 2.6	3.7
PEG(4500) G-CSF(2)	6	17.5 \pm 3.0	3.1
<Exp. II>			
vehicle	6	12.3 \pm 1.7	1.0
control G-CSF	6	27.1 \pm 4.6	2.2
PEG(4500) G-CSF(3)	6	54.0 \pm 7.2	4.4
b. 100 $\mu\text{g/kg}$			
<Exp. I>			
vehicle	6	6.6 \pm 0.7	1.0
control G-CSF	6	18.5 \pm 2.3	2.8
PEG(4500) G-CSF(1)	6	42.9 \pm 4.3	6.5
PEG(4500) G-CSF(2)	6	22.6 \pm 1.9	3.4

EXAMPLE 6

In vivo biological assay of PEG (4,000) G-CSF

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,000) G-CSF obtained in EXAMPLE 4. Samples of the intact human G-CSF and PEG (4,000) G-CSF were intravenously injected into mice at a dose of 10 μg protein/kg. At 24 hrs after the injection, blood was collected from orbital vein and the number of neutrophils was counted as in EXAMPLE 5. The results are shown in TABLE 3.

It has been revealed that PEG (4,000) G-CSF in which the activated PEG is bound through the free carboxyl group has also increased the number of neutrophils more than the intact human G-CSF has.

TABLE 3

Pharmacological activity (in vivo) of PEG (4,000) G-CSF			
Group	Number of Animals	Number of Neutrophils ($\times 10^2 / \mu\text{l}$)	Ratio (to vehicle)
Vehicle	6	10.9 + 1.0	1.0
G-CSF (control)	6	16.4 + 1.4	1.5
PEG (4,000) G-CSF	6	23.3 + 2.5	2.1

EXAMPLE 7

Increasing Effects of PEG-modified human G-CSFs on mice neutrophils

Male ICR mice (7 weeks old) were used for *in vivo* assays for pharmacological activity of PEG (4,500) G-CSF and PEG (10,000) G-CSF obtained in EXAMPLES 1 and 3, respectively. PEG (4,500) G-CSF used here is a high molecular fraction from DEAE ion-exchange chromatography of a product obtained in the reaction wherein the molar ratio of the activated PEG / the free amino group was fifty (an average molecular weight of 60K; 38K:20%, 58K:54%, 80K:27%). Samples of the human G-CSF, PEG (4,500) G-CSF and PEG (10,000) G-CSF were intravenously injected into mice at a dose of 10 μg protein/kg. At 6, 24, 32, 48 and 72 hrs after the injection, blood was collected from orbital vein and the

number of neutrophils was counted as in EXAMPLE 5, except for using an auto blood cell counter CC180-A (Toa Medical Electronics, Japan).

As shown in FIG.2, in the case of the intact human G-CSF, the number of neutrophils decreases to a basal level 24 hrs after the injection. On the other hand, a significant increase of neutrophils was observed over 32 hrs and 48 hrs after the injection for PEG (4,500) G-CSF and PEG (10,000) G-CSF, respectively.

Moreover, male ICR mice (8 weeks old) were intravenously administered with the PEG (10,000) G-CSFs obtained in EXAMPLE 3; (a) an average molecular weight of 30 K, (b) an average molecular weight of 51 K; 40K:58%, 66K:42% at a dose of 10 µg protein/kg. At 24 hours after the injection the number of neutrophils was counted as in EXAMPLE 5. The results are shown in TABLE 4.

TABLE 4

Pharmacological activity (in vivo) of PEG (10,000) G-CSF			
Group	Number of Animals	Number of Neutrophils (x 10 ² / µl)	Ratio (to vehicle)
Vehicle	5	7.4 ± 0.6	1.0
G-CSF	5	16.4 ± 3.1	2.2
PEG(10,000) G-CSF (a)	5	68.9 ± 10.5	9.3
PEG(10,000) G-CSF (b)	5	95.8 ± 6.4	12.9

Both PEG (10,000) G-CSF (a) and (b) have increased the number of neutrophils more than the intact human G-CSF has. Especially, PEG (10,000) G-CSF with a higher extent of the modification showed a more remarkable increase in the number of neutrophils, just like PEG (4,500) G-CSF did.

EXAMPLE 8

Effects of PEG-modified human G-CSF on cyclophosphamide-induced neutropenic mice

Male ICR mice (7 weeks old) were intraperitoneally injected with 200 mg/kg cyclophosphamide (CY) to induce neutropenia. Once a day for successive 4 days starting from one day after the CY injection, PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 were intravenously injected into the neutropenic mice at a dose of 10 µg protein/kg. At 6, 24 and 48 hrs after the last injection, blood was collected from orbital vein and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.3, PEG-modified G-CSFs have accelerate the recovery from neutropenia induced by the injection of cyclophosphamide similar or earlier than the intact G-CSF. Especially, PEG (10,000) G-CSF has effected a significant increase in the number of neutrophils.

EXAMPLE 9

Effects of PEG-modified human G-CSF on 5-FU-induced neutropenic mice

Female BDF₁ mice (7 weeks old, JAPAN SLC Co.,) were intravenously injected with 200 mg/kg 5-FU to induce neutropenia. At a dose of 10 µg protein/kg once a day for successive 11 days (PEG-1), for every other day (at day 1, 3, 5, 7, 9 and 11; PEG-2) and every third day (at day 1, 4, 7 and 10; PEG-3) starting from one day after the 5-FU injection, the same PEG (10,000) G-CSF as used in EXAMPLE 7 and the intact human G-CSF were subcutaneously injected into the neutropenic mice. At day 7, 8, 9, 10, 11, 12, 14 and 17, blood was collected from orbital vein and neutrophils were counted as in EXAMPLE 5.

As shown in FIG.4, it took about 14 days to recover neutrophil counts of mice injected with only 5-FU to a basal level. On the other hand, it took about 11 days and 9 days to recover neutrophil counts of mice injected also with the intact human G-CSF, and PEG-1, 2 and 3, respectively. Thus, PEG-modified G-CSFs have accelerated the recovery from neutropenia induced by the injection of 5-FU earlier than the intact G-CSF. Moreover, even with fewer times of injection of the PEG-modified G-CSFs than the intact human G-CSF, the same phenomena as the above could be observed.

EXAMPLE 10Acute toxicity of PEG-modified human G-CSF

5 Male and female Slc:IR mice (5 weeks old) groups consisting 6 mice each were intravenously administered with the same PEG (4,500) G-CSF and PEG (10,000) G-CSF as used in EXAMPLE 7 as well as vehicles at a dose of 12 ml/kg. General conditions and survival of the treated mice were observed as often as possible for 6 hrs immediately after administration and once a day for the following 14 days. The body weight was checked at the day of injection, 5, 8, 12 and 15th days. Surviving mice were bled to death under ether anesthesia and subjected to pathologic autopsy.

10 As shown in TABLE 5, no mouse died for the observed period. LD 50 for both PEG (4,500) G-CSF and PEG (10,000) G-CSF was estimated over 3,000 µg protein/kg in both male and female mice. No remarkable change in general condition, body weight or opinion of the autopsy was observed for PEG (4,500) G-CSF or PEG (10,000) G-CSF. These results may suggest that the acute toxicity of PEG-modified human G-CSF is very weak, as the intact human G-CSF is .

TABLE 5 Mortality of male and female mice

Sex	Compound	Dose ($\mu\text{g/kg}$)	Number of deaths on day															Mortality [†]	LD ₅₀ ($\mu\text{g/kg}$)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Male	Vehicle	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	—
	PEG4500-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
	PEG10000-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
Female	Vehicle	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	—
	PEG4500-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000
	PEG10000-G-CSF	3,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/6	>3,000

† : No. of dead animals / No. of treated animals

EXAMPLE 11**Determination of half-life of PEG-modified hG-CSF**

5 Male Sprague-Dawley rats (7 weeks old) were used for study of pharmacokinetics of the intact human G-CSF and PEG (10,000) G-CSF prepared in EXAMPLE 3. Samples were intravenously injected into rats at a dose of 100 µg protein/kg. At 10 min, 2, 4, 8, 24 and 48 hrs after the injection, about 6 -7 ml of blood of each of three rats was collected from abdominal aorta into a polypropylene tube of about 15 ml volume and centrifuged (18,000 x g) at 4°C for 5 min to prepare a serum fraction. An amount of the active human G-CSFs contained in the serum fraction was determined by
10 a bioassay for proliferation induction of mouse bone marrow cells on the basis of incorporation of ³H-thymidine (Ralph et al., Blood 66, pp.633-639, (1988)). The time course of serum concentration is shown in FIG. 5. The results indicate that the half lives of the intact human G-CSF and PEG (10,000) G-CSF are 1.79 hrs and 7.05 hrs, respectively, and AUCs are also 2,000 ng protein hrs/ml and 16,195 ng protein hrs/ ml, respectively. Accordingly, it is demonstrated that the clearance rate of PEG (10,000) G-CSF in the body has been decreased more than that of the intact human G-CSF
15 has.

Industrial Applicability

20 It is expected that the present PEG-modified human G-CSF may make a great contribution to the treatment with human G-CSF because it has a neutrophils-increasing activity much more lasted than that of the intact human G-CSF, enabling fewer numbers of administration with a lower dose.

Claims

- 25 1. A chemically-modified protein prepared by binding polyethylene glycol to a polypeptide characterized by being the product of expression by a host cell of an exogenous DNA sequence and substantially having the following amino acid sequence, through a carboxyl group of the amino acid(s) of the polypeptide:
- 30
- 35
- 40
- 45
- 50
- 55

(Het)n

5 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln.
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 10 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 15 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 20 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 25 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 30 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
 Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
 35 Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 40 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 Arg Val Leu Arg His Leu Ala Gln Pro

(n=0 or 1)

- 50 2. The chemically-modified protein according to Claim 1 in which at least one aspartic acid or glutamic acid is included.
3. A protein according to Claim 1 or 2 for use as a medicament.
4. A protein according to Claim 1 or 2 for use in the treatment of neutropenia.
- 55 5. A pharmaceutical composition comprising a protein according to Claim 1 or 2, a pharmaceutically acceptable diluent, an agent for preparing an isotonic solution and a pH-conditioner.

Patentansprüche

1. Ein chemisch modifiziertes Protein, das hergestellt wird durch Binden von Polyethylenglycol an ein Polypeptid, das dadurch gekennzeichnet ist, daß es das durch eine Wirtszelle exprimierte Produkt einer exogenen DNA-Sequenz ist und im wesentlichen die folgende Aminosäuresequenz aufweist, durch eine Carboxylgruppe der Aminosäure (n) des Polypeptids:

(Het)n

10 Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln.
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 15 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 20 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 25 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 30 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 35 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
 Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
 40 Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 45 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 50 Arg Val Leu Arg His Leu Ala Gln Pro (n = 0 oder 1)

2. Das chemisch modifizierte Protein nach Anspruch 1, in dem wenigstens eine Asparaginsäure oder Glutaminsäure enthalten ist.
3. Ein Protein nach Anspruch 1 oder 2 zur Verwendung als ein Medikament.
4. Ein Protein nach Anspruch 1 oder 2 zur Verwendung bei der Behandlung von Neutropenie.

5. Eine pharmazeutische Zusammensetzung, die ein Protein nach Anspruch 1 oder 2, ein pharmazeutisch akzeptables Verdünnungsmittel, ein Agens zur Herstellung einer isotonischen Lösung und einen pH-Zusatzstoff umfaßt.

Revendications

1. Protéine modifiée chimiquement préparée par liaison de polyéthylène glycol à un polypeptide, caractérisée en ce qu'elle est le produit d'expression par une cellule hôte d'une séquence d'ADN exogène et a pratiquement la séquence d'acides aminés suivante, par l'intermédiaire d'un groupe carboxy du ou des acides aminés du polypeptide :

(Het)n

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
 Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
 Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
 Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
 Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
 Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
 Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
 Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
 Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Het
 Glu Glu Leu Gly Het Ala Pro Ala Leu Gln Pro
 Thr Gln Gly Ala Het Pro Ala Phe Ala Ser Ala
 Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
 Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
 Arg Val Leu Arg His Leu Ala Gln Pro

(n = 0 ou 1)

2. Protéine modifiée chimiquement suivant la revendication 1, dans laquelle au moins un acide aspartique ou un acide

glutamique est inclus.

3. Protéine suivant les revendications 1 ou 2, utilisable comme médicament.
- 5 4. Protéine suivant les revendications 1 ou 2, utilisable dans le traitement de la neutropénie.
5. Composition pharmaceutique comprenant une protéine suivant les revendications 1 ou 2, un diluant acceptable du point de vue pharmaceutique, un agent pour préparer une solution isotonique et un agent pour ajuster le pH.

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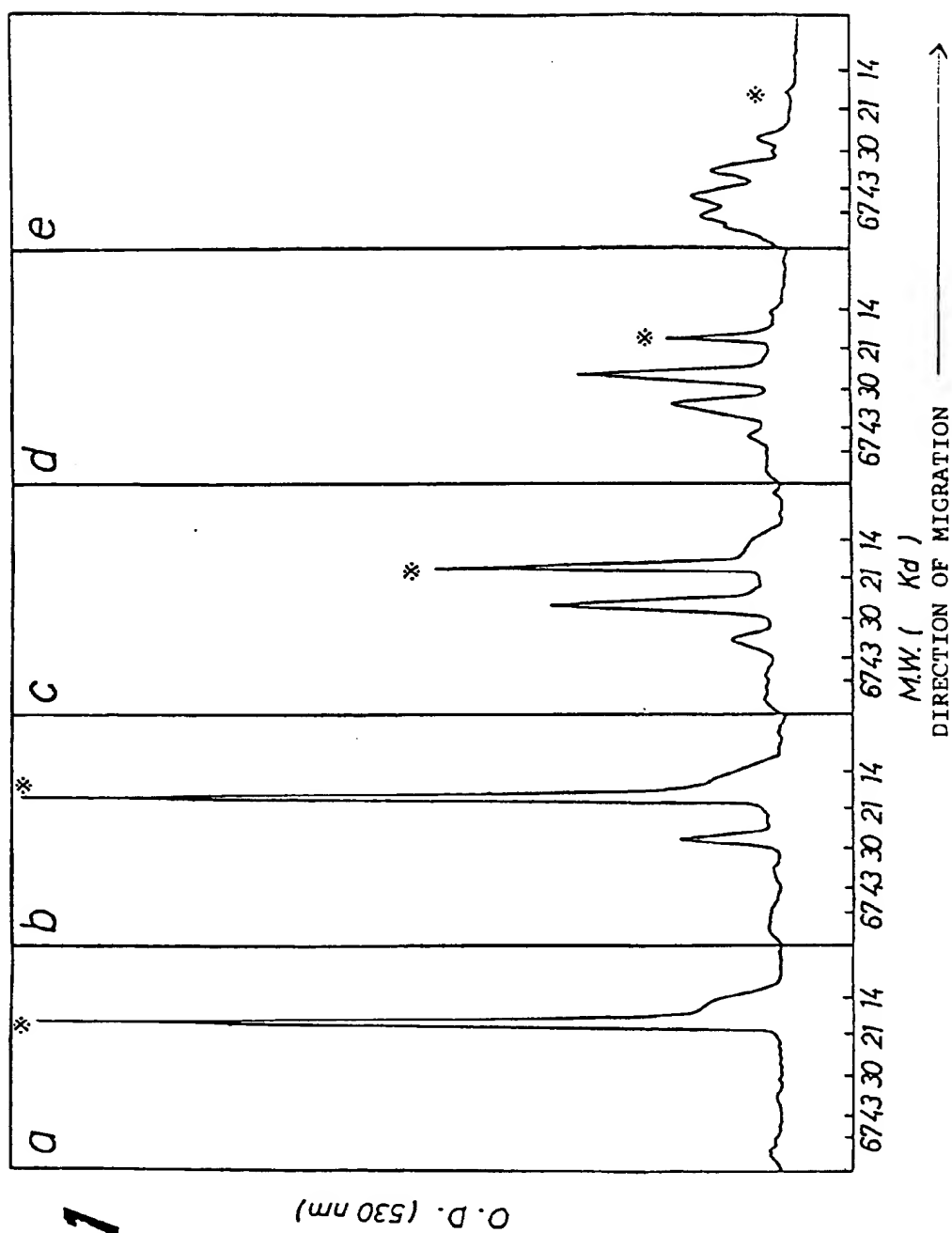


FIG. 2

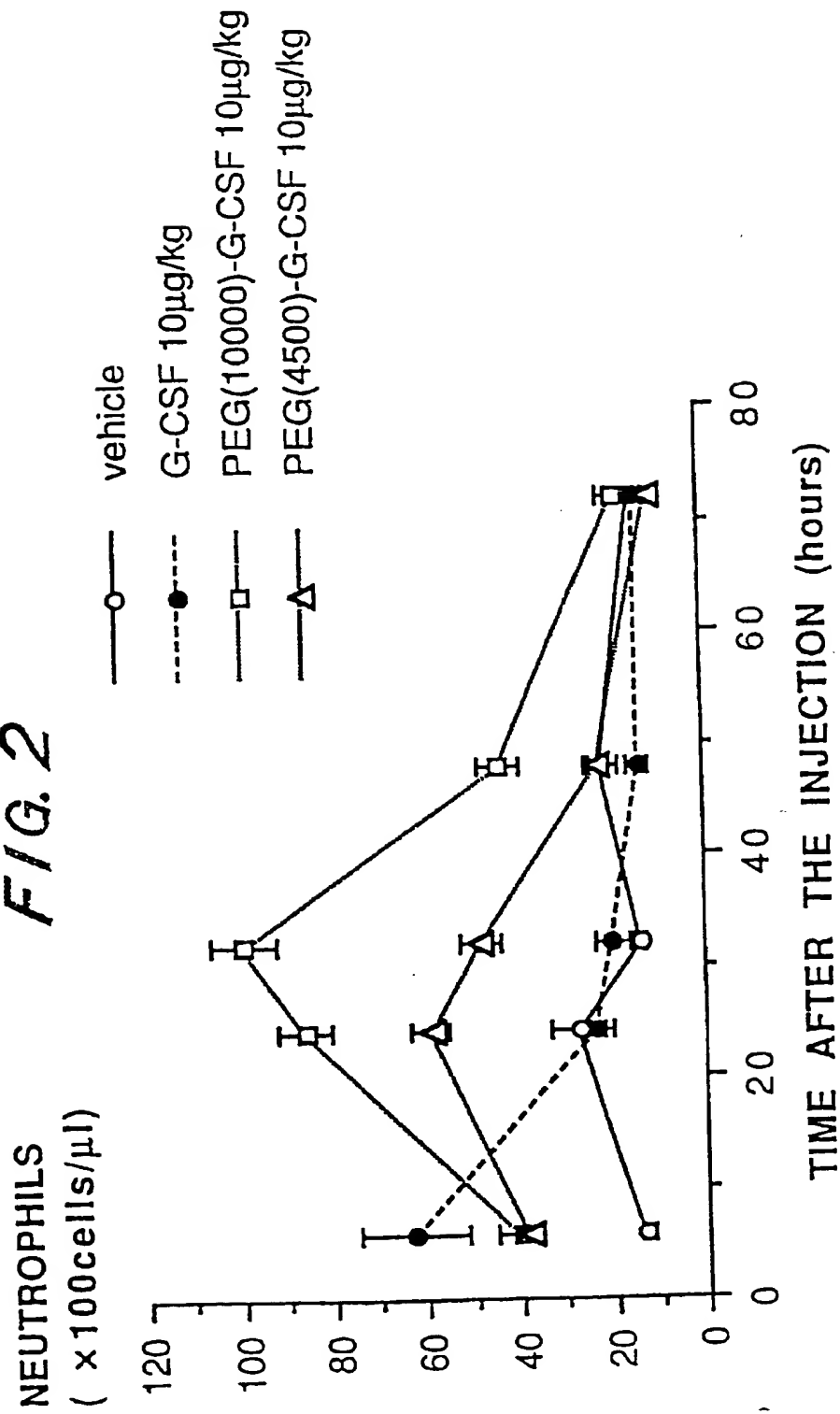
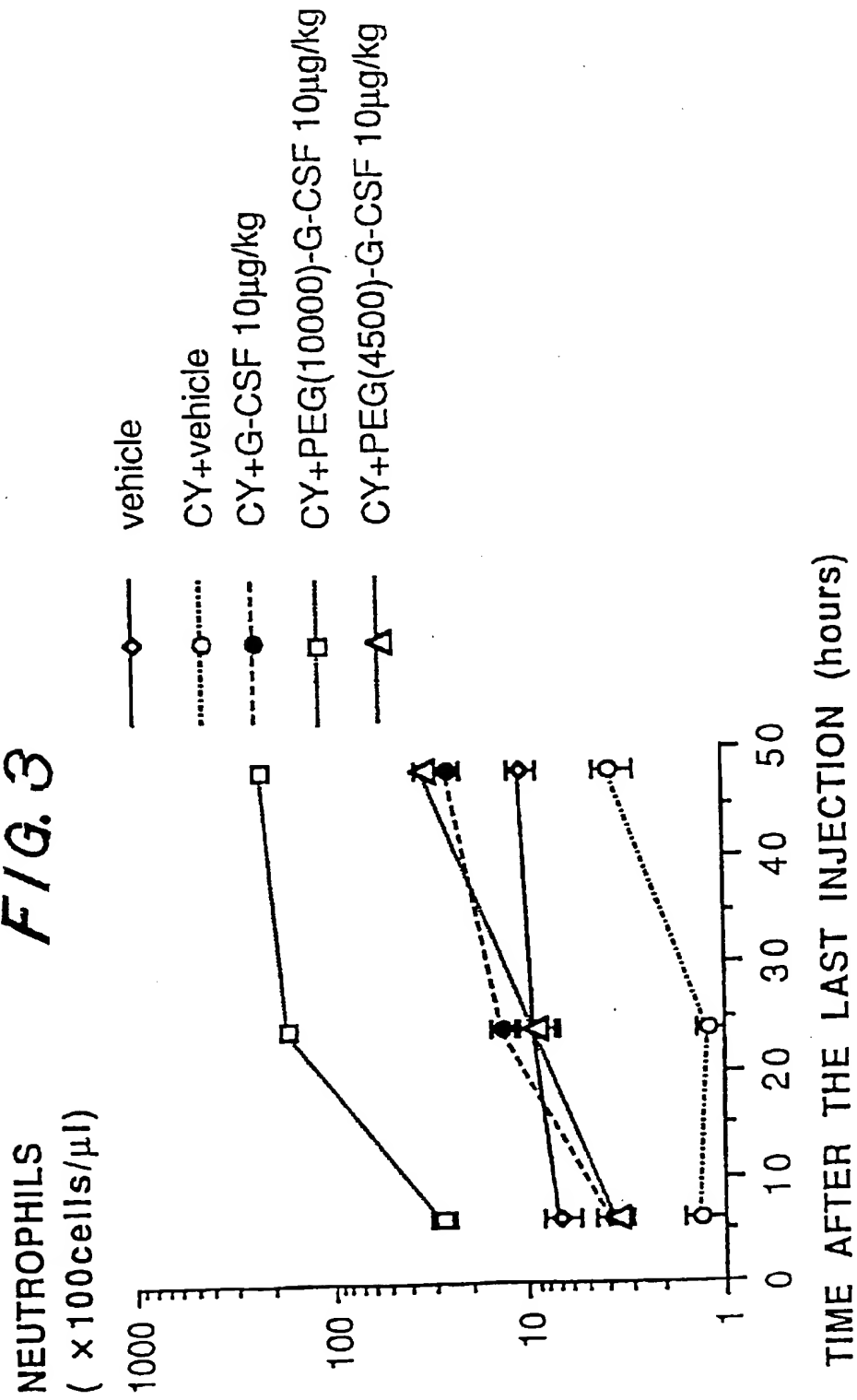


FIG. 3



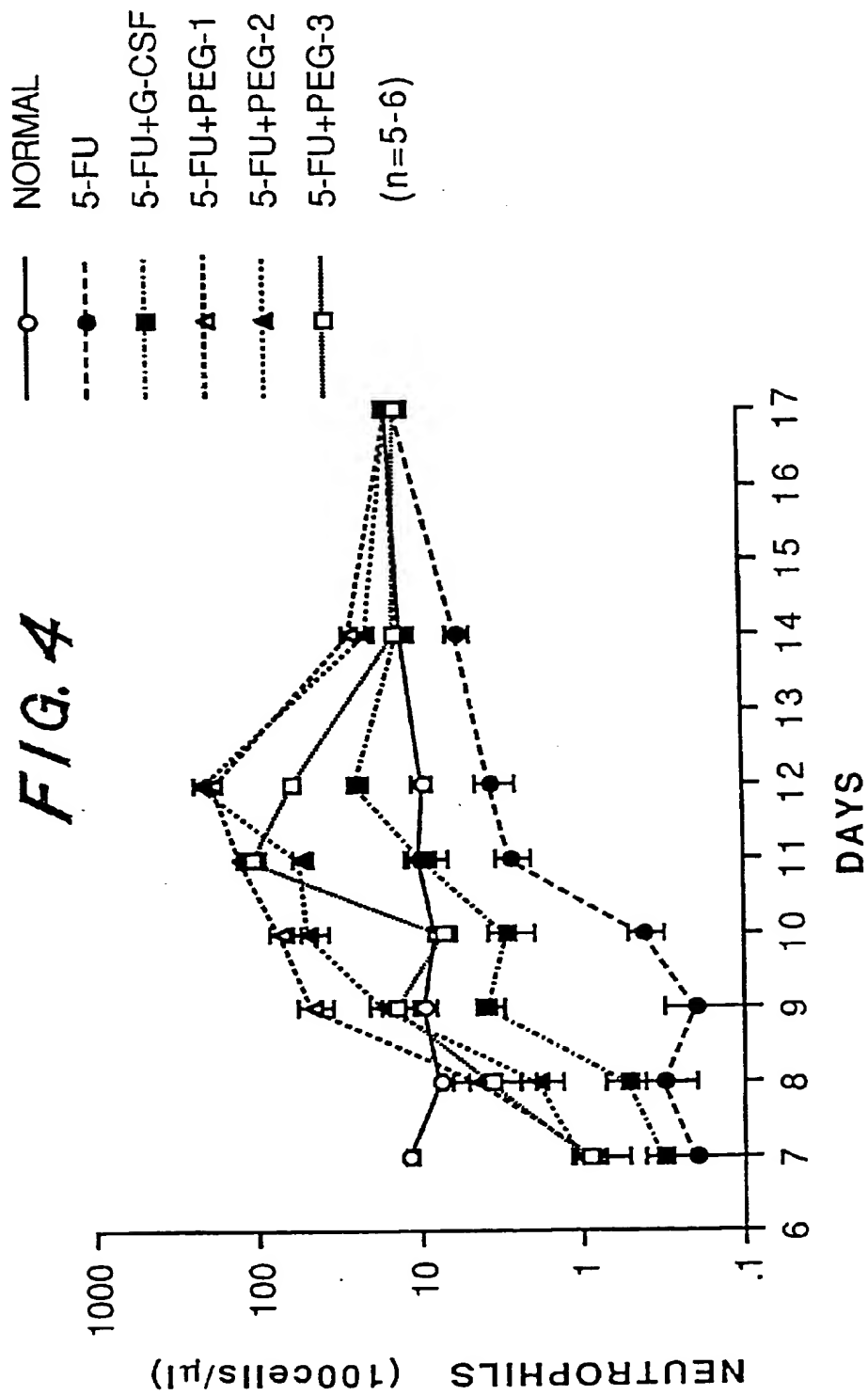


FIG. 5